Optically trapped aqueous droplets for single molecule studies

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We demonstrate a technique for creating, manipulating, and combining femtoliter volume chemical containers. The containers are surfactant-stabilized aqueous droplets in a low index-of-refraction fluorocarbon medium. The index-of-refraction mismatch between the container and fluorocarbon is such that individual droplets can be optically trapped by single focus laser beams, i.e., optical tweezers. Here, we trap and manipulate individual droplets, detect the fluorescence from single dye and red fluorescent protein molecules encapsulated in droplets, and observe fluorescence resonance energy transfer from a single dye pair on a deoxyribonucleic acid molecule encapsulated in a droplet. © *2006 American Institute of Physics*. [DOI: 10.1063/1.2219977]

Techniques for optically observing single molecules are changing our understanding of molecular processes in biology.[1] Measurements can be made on single molecules in a dilute solution as they diffuse through the measurement volume; however, the dynamics of a single molecule cannot be followed for longer than the transit time, typically less than a few milliseconds.[2] For longer times, methods that immobilize or confine the molecule or molecular complex under study, while still permitting physiological interactions, are used. Current immobilization strategies include binding[3–5] or adsorption[6–9] of molecules on a surface or in porous materials.[10,11] For such strategies, the role of surface interactions is always an issue. Femtoliter-sized containers can confine single molecules inside the detection volume typical of a confocal microscope,[12] yet allow the molecule to freely diffuse for a substantial amount of time. This confinement strategy also more closely mimics the freely diffusing but confining environment in which many biochemical processes take place within a cell.

This letter describes the creation, individual manipulation, and application of femtoliter aqueous droplets in an immiscible fluorocarbon liquid for confining, isolating, and interrogating individual molecules or molecular complexes. This approach is similar to, but offers some simplifications and advantages over, previous work on single biomolecules encapsulated in surface-tethered liposomes (lipid vesicles).[12–14] First, the formation and purification of aqueous droplet-encapsulated molecules is simple. The formation of droplets can be accomplished by the ultrasonic mixing of an aqueous buffer solution containing the species of interest and the matrix fluorocarbon. Encapsulation in liposomes requires the lipid membrane to surround the analyte molecules in the aqueous phase. Therefore, a purification step is necessary to remove the hydrophilic analyte molecules from the aqueous matrix outside the liposome. No purification step is necessary in our approach to eliminate hydrophilic molecules from the hydrophobic fluorocarbon matrix; the molecules naturally partition into the aqueous droplets. Second, our droplets can be optically trapped and held stationary without the need for surface attachment chemistry. Third, optically trapped droplets can also be brought into contact with each other and, unlike liposomes, spontaneously fuse so mixing can be performed quickly and easily.

We use a single focus laser trap (optical tweezers) to trap and remotely manipulate microscopic aqueous droplets in a fluorocarbon background medium. Optical tweezers rely on the increased polarizability of the object to be trapped compared to the surrounding medium, such that the energy of interaction between the object and the laser field is a minimum. That is, the object to be trapped must have an index of refraction higher than the surrounding medium. The index of refraction of water is 1.33; for the fluorocarbon, FC-77 (3M), it is 1.29. Therefore, the droplets are easily trapped with optical tweezers.

Figure 1 illustrates some features of the optical trapping setup, which has been described in detail elsewhere[15] that allow for single molecule fluorescence detection. One of the optical trapping beams overlaps a green laser beam (HPM-150, Extreme Lasers) that was defocused to $\approx 5\ \mu$m to approximate uniform illumination of the 1 μm diameter spot at the focus of the trapping laser. Light collected by the objective (100×, 1.4 NA, Zeiss) was focused onto a 100 μm pinhole, which rejects background light outside of an $\approx 0.2\ \mu\text{m}^3$ focal volume. The light from the pinhole was filtered (XB11, Omega Optical and HQ550LP, Chroma Technology) to allow only the fluorescence of interest to be focused down onto the active area (diameter=175 μm) of a photon-counting avalanche photodiode (APD) (SPCM-AQR-14, Perkin Elmer). In the case of single pair fluorescence resonance energy transfer (spFRET), detection mirror M3 was replaced with a dichroic mirror (XF2021, Omega Optical) followed by a fluorescent filter (3RD650LP, Omega Optical) and a second APD. Both APDs sent digital pulses to a counting card (NCI-6602, National Instruments) controlled by software (LABVIEW 6.1, National Instruments). Photons were counted in 1 ms time bins.

Several different aqueous solutions were prepared. Red fluorescent protein (rDs Red2 protein, BD Biosciences Clontech) was mixed with phosphate buffer solution (Sigma), 10 mmol/L ethylenediaminetetaacetic acid (EDTA) (Sigma),

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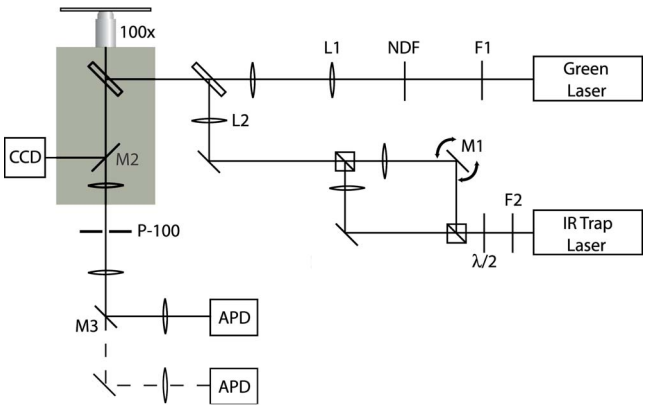

FIG. 1. (Color online) A schematic diagram of the optical setup. F1 and F2 are bandpass filters for 532 nm and 1064 nm light, respectively. NDF is a series of neutral density filters. L2 is the second lens of the IR trap beam telescope that is mounted on a three-axis translation stage for precisely overlapping the trap and fluorescence beams. M1 is the movable mirror for the moving optical trap in the fusion demonstration. All elements in the shaded gray region are internal to an inverted microscope (Axiovert 100, Zeiss) and M2 is a manual flipper mirror that directs light to the charge coupled device camera or is removed to allow passage to the APD. P-100 is a 100 μm pinhole that is part of the confocal microscope setup. FRET measurements were made by replacing the M3 mirror with a dichroic mirror, and a second APD was inserted to capture light from the acceptor fluorophore. Also, the P-100 pinhole was removed for the FRET measurement to avoid issues due to chromatic aberrations.

and 10% glycerol at *p*H=7.45. Sulforhodamine B (SRB) (Sigma) and fluorescently labeled single-stranded deoxyribonucleic acid (DNA) (16mer ssDNA, Sigma-Aldrich) were diluted in a tris(hydroxymethyl)aminomethane (TRIS) (Gibco) buffer containing an oxygen scavenging system that consisted of 4% beta-mercaptoethanol (Pierce), 50 μg/mL glucose oxidase (Roche Diagnostics Co.), 10 μg/mL catalase (Roche Diagnostics Co.), 18% (w:w) glucose in a 10 mmol/L TRIS buffer with 150 mmol/L NaCl and 2 mmol/L EDTA at *p*H 7.5.

Each sample was prepared by mixing 100 μL of aqueous solution with a surfactant (Triton X-100, Sigma) at 0.1% by volume. This was then added to 1 mL of the FC-77, which was filtered down to 0.2 μm. Ultrasonicating (Branson 5510) this mixture, at room temperature for solutions containing SRB or DNA and in an ice bath for red fluorescent protein (RFP), for 30 s produced stable (the solubility of water in FC-77 at room temperature is a few ppm) aqueous droplets, less than 2 μm in diameter.

A chamber, consisting of a microscope slide with a hole drilled through it sandwiched between two coverslips,[15] contained a collection of droplets that we observed under bright-field illumination. The sample was manually translated until the optical tweezers trapped a single droplet approximately 5 μm above the coverslip surface. The bright-field illumination was then blocked and the optical path to the APD was opened. After 2 s–4 s, a mechanical shutter on the green laser path was unblocked. Fluorescence was collected until all the dye molecules in the droplet were photobleached, typically 5 s–20 s. The concentration of droplets was kept low enough so that no other droplets were trapped during this time. The droplet was then released from the trap, and the process repeated with a fresh droplet.

Figure 2 shows three measurements of the fluorescence from single SRB dye molecules contained in different aqueous droplets. The first few seconds show dark counts from the detector (about 100 counts/s), and the initial jump in intensity occurs when the shutter on the green laser path is opened, producing a combination of fluorescence emission and laser background counts. The counts irreversibly decrease in discrete steps until only dark counts, plus the background from the excitation light, are measured. The discrete drops in emission are indicative of the photobleaching of individual dye molecules, and the number of dye molecules in the droplet can be determined by the number of photobleaching steps.[12] Thus, Figs. 2(a)–2(c) show results from droplets with one, two, and three dye molecules, respectively. Results similar to those in Fig. 2 were obtained for free Cy3 dye, Cy3 attached to a DNA strand, and tetramethylrhodamine (TMR) attached to a DNA strand.

To demonstrate that more fragile biomolecules remain functional during the droplet formation and trapping process, we encapsulated RFP. Fluorescence from single RFP molecules in an optically trapped droplet was observed.[21] To estimate the survival probability of RFP during the ultrasonication process, we measure the fluorescence from droplets containing RFP at 360 nmol/L. The setup was modified so that the focus of the green excitation laser coincided with the trapped droplets, and we removed the 100 μm pinhole before the single APD. If the RFP is not damaged during the ultrasonication process then we expect to see equal fluorescence intensity from bulk solution and from a large trapped droplet ($D \approx 2$ μm) having equal concentrations of RFP. After correcting for detection efficiencies, determined by a control experiment,[21] we find that at least 50% of RFP survive the ultrasonic agitation.

To demonstrate the feasibility of spFRET studies, we encapsulate single-stranded 16mer DNA molecules with the FRET pair Cy3 (the donor), attached to the 5′ end, and Cy5 (the acceptor), attached to the 3′ end. The nominal length of the 16mer was less than the Förster radius for the FRET pair; therefore, nonradiative energy transfer from the excited donor dye molecule to the acceptor dye molecule occurs. The green laser excites the FRET pair, which results in the

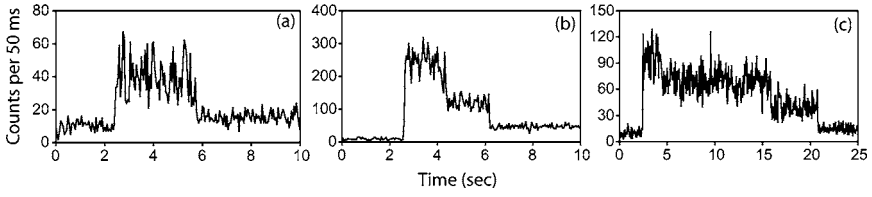

FIG. 2. (a)–(c) Three examples of single molecule detection in trapped droplets illustrating the trapping and detection of 1, 2, and 3 (SRB) molecules, respectively. The measurements are taken with different excitation strengths. For (a) and (c), the laser power sent into the back aperture of the microscope objective was 600 μW, and for (b) the power used was 2.5 mW. The different laser powers resulted in different step sizes for photobleaching events. For these measurements, a solution of 5 nmol/L SRB was used, such that a 1 μm droplet is calculated to contain an average of 1.6 dye molecules.

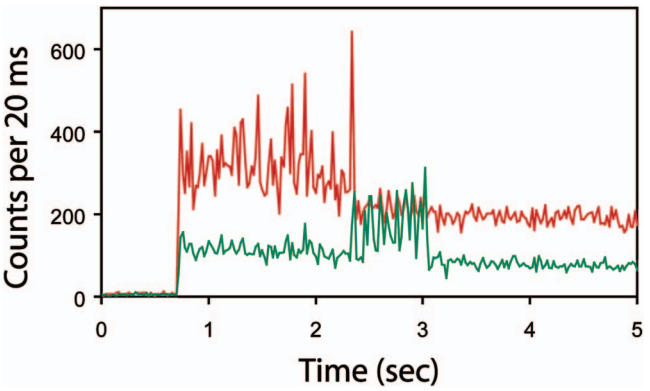

FIG. 3. (Color) Single molecule FRET data obtained from a single-stranded 16mer DNA with a Cy3 molecule attached to the 5′ end and a Cy5 molecule attached to the 3′ end in an optically trapped droplet. The Cy3 donor molecule is quenched by the Cy5 acceptor molecule until the photobleaching of the Cy5 molecule occurs at $t=2.3$ s. The donor molecule fluoresces until $t=3.0$ s when the donor molecule photobleaches.

quenching of the Cy3 and fluorescence from the Cy5. Figure 3 is an example of a measured spFRET trajectory.

Aqueous droplets are well suited for experiments that require mixing. This includes nonequilibrium experiments—in which the environment needs to be changed rapidly—and experiments involving individual transiently interacting molecular complexes. (For example, most nucleic acid/protein complexes have short lifetimes and, therefore, are not readily studied on an individual basis.) Figure 4 is a sequence of video images showing the fusion of two droplets held in independent optical tweezers.[21] Fusion of aqueous droplets differs from that of vesicles in two major ways. First, droplets fuse spontaneously when they are brought into contact,[16] unlike vesicles where the fusion of two independent vesicles has to be induced by an external device such as a microelectrode[17] or a pulsed laser beam.[15] Second, if molecules do not partition into the FC-77 (i.e., no leakage), then there will be no loss of encapsulated reagent during the fusion process.

Studies of kinetics rely on accurate measurements of volumes and concentrations. The uncertainty in measuring droplet sizes is an obstacle that must be overcome in order to perform mixing of different concentrations of reagent. Recent work by Link *et al.*[18] demonstrates the creation of monodisperse water droplets using microfluidic channels;[18] however, these droplets are too large for single molecule studies. Other groups have created drops of only a few microns in diameter with micropipettes and piezoelectric actuators.[19,20] Micron-sized droplet creation is an active area of research, and it seems likely that methods for creating very small droplets to suit many different applications will be available shortly.

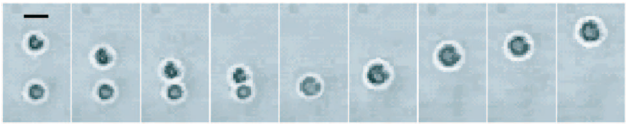

FIG. 4. (Color) Sequence of video images showing the fusion of two aqueous droplets, initially held in independent optical tweezers. The upper droplet is translated by the mobile trap to the location of the droplet held by the fixed trap, at which point the two droplets fuse into one. The fixed trap is then turned off and the single droplet is translated upward by the mobile trap. The mobile trap (upper) is slightly defocused from the fixed trap (lower). The solid bar in the first picture is 1 μm in length.

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[1] S. Weiss, Nat. Struct. Biol. **283**, 1676 (1999).
[2] M. Eigen and R. Rigler, Proc. Natl. Acad. Sci. U.S.A. **91**, 5740 (1994).
[3] T. Ha, X. Zhuang, H. D. Kim, J. W. Orr, J. R. Williamson, and S. Chu, Proc. Natl. Acad. Sci. U.S.A. **96**, 9077 (1999).
[4] S. Wennmalm, L. Edman, and R. Rigler, Proc. Natl. Acad. Sci. U.S.A. **94**, 10641 (1997).
[5] H. Noji, R. Yasuda, M. Yoshida, and K. Kinosita, Nature (London) **386**, 299 (1997).
[6] Y. Jia, D. S. Talaga, W. L. Lau, H. S. M. Lu, W. F. DeGrado, and R. M. Hochstrasser, Chem. Phys. **247**, 69 (1999).
[7] D. S. Talaga, W. L. Lau, H. Roder, J. Y. Tang, Y. Jia, W. F. DeGrado, and R. M. Hochstrasser, Proc. Natl. Acad. Sci. U.S.A. **97**, 13021 (2000).
[8] M. A. Bopp, Y. W. Jia, L. Q. Li, R. J. Cogdell, and R. M. Hochstrasser, Proc. Natl. Acad. Sci. U.S.A. **94**, 10630 (1997).
[9] T. Wazawa, Y. Ishii, T. Funatsu, and T. Yanagida, Biophys. J. **78**, 1561 (2000).
[10] R. M. Dickson, A. B. Cubitt, R. Y. Tsien, and W. E. Moerner, Nature (London) **388**, 355 (1997).
[11] H. P. Lu, L. Y. Xu, and X. S. Xie, Science **282**, 1877 (1998).
[12] E. Boukobza, A. Sonnenfeld, and G. Haran, J. Phys. Chem. B **105**, 12165 (2001).
[13] E. Rhoades, E. Gussakovsky, and G. Haran, Proc. Natl. Acad. Sci. U.S.A. **100**, 3197 (2003).
[14] B. Okumus, T. J. Wilson, D. M. J. Lilley, and T. Ha, Biophys. J. **87**, 2798 (2004).
[15] S. Kulin, R. Kishore, K. Helmerson, and L. Locascio, Langmuir **19**, 8206 (2003).
[16] K. T. Kotz, K. A. Noble, and G. W. Faris, Appl. Phys. Lett. **85**, 2658 (2004).
[17] D. T. Chui, C. F. Wilson, F. Ryttsen, A. Stromberg, C. Farre, A. Karlsson, S. Nordholm, A. Gaggar, B. P. Modi, A. Moscho, A. Garza-Lopez, O. Orwar, and R. N. Zare, Science **283**, 1892 (1999).
[18] D. R. Link, S. L. Anna, D. A. Weitz, and H. A. Stone, Phys. Rev. Lett. **92**, 054503 (2004).
[19] W. H. Zhang, L. Hou, L. Mu, and L. Zhu, Proc. SPIE **5345**, 220 (2003).
[20] C. Lee and A. Lal, IEEE Trans. Ultrason. Ferroelectr. Freq. Control **51**, 1514 (2004).
[21] See EPAPS Document No. E-APPLAB-89-216628 for material on fluorescence from single RFP molecules, the control experiment to determine RFP survival probability, and a video of controlled droplet fusion. This document can be reached via a direct link in the online article's HTML reference section or via the EPAPS homepage (http://www.aip.org/pubservs/epaps.html).